

# Effects of in vitro Exposure of Human Respiratory Epithelial Cells to Formaldehyde

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## **Abstract**

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(under direction of Dr. Harvey E. Jeffries)

Formaldehyde is the most abundant carbonyl in air. As a by-product of combustion and other natural processes, it is ubiquitous in the environment. Typically, the formaldehyde concentration levels in ambient air are produced by the photochemical oxidation of volatile organic compounds (VOCs), including the major biogenics such as isoprene and methane. Thus, formaldehyde is an important component of atmospheric pollution. Also, it is commonly used in the medical and health fields, in the manufacturing of building materials, and is present in substantial quantities in occupational and home environments. People are likely exposed to low levels of formaldehyde on a daily basis, it has therefore been suggested that formaldehyde exposure may be linked to increased hospital admissions and increased prevalence of inflammatory diseases. Other carbonyl compounds similarly produced and measured in the atmosphere have been shown to produce measurable cytotoxic and inflammatory responses. However, it is largely unknown how exposure to formaldehyde effects specific inflammatory mediator production. To evaluate the potential adverse health effects elicited by formaldehyde exposure cultured human respiratory epithelial cells (A549) were used as the target of the formaldehyde gas. In cell culture supernatants, cellular distress

was measured by the production of pro-inflammatory mediators such as interleukin-8 and Interleukin-6. Cytotoxicity or cellular death was measured by the release of lactate dehydrogenase (LDH). Using permeation tube or large chamber air mixtures, formaldehyde was administered in increasing doses to investigate a dose response of concentrations that have been observed in ambient and occupational environments. Formaldehyde concentrations were measured continuously using a permeable membrane sampler, and detected with fluorometry using an automated Hantzsch derivative reaction (Dasgupta technique). The concentrations in the 3-hr exposures were approximately 55 ppb, 140 ppb, 220 ppb, 390 ppb, and 980 ppb. At the 55 to 220 ppb formaldehyde exposure level, there were virtually no differences between exposed cells and control cells for all endpoints. Cells exposed to 390 and 980 ppb showed no increases in LDH or IL-6, however a concentration dependent decrease was observed in IL-8 expression for both chamber and permeation tube exposures. This data suggests that gaseous formaldehyde does not affect common markers of inflammation or cause cellular death in lung epithelial cells. However the observed decrease in IL-8 expression implies that there may be a mechanism, unknown to us, by which formaldehyde is suppressing the release of IL-8 proteins.

*To my parents, and to Sheri, Randy, and family, my accomplishments are merely a reflection of the unconditional love, support, and guidance you have given me over the years. You have given me the courage to follow my dreams and the fortitude to accept no limitations. To my sister, you never cease to amaze me and I wish we lived much closer!*

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# 1 Background

Over the past two decades an unsettling trend in epidemiologic data indicates that the prevalence of asthma and other allergic airway diseases is increasing at alarming rates (Beasley et al). Although there is evidence that air pollution may be the leading cause, the scientific community has not been able to establish an obvious chemical or pollutant as primarily responsible. It has therefore been suggested that formaldehyde, a chemical compound that is widely distributed in the environment and one that can affect a large portion of the population, may be a cause of the increasing prevalence of chronic respiratory diseases (Li Guang-Young, 2007).

## 1.1 Formaldehyde

Formaldehyde (HCHO) is an organic chemical containing only one terminal carbonyl group. It is the simplest aldehyde and the most abundant carbonyl in air. It is a chemical of enormous economic impact; estimates from 1992 indicate that over 12 million tons of formaldehyde are produced worldwide. In the U.S. on a daily basis approximately 1.5 million workers are exposed to formaldehyde, and about 1 million in the European Union (Minisymposium 1: Occup. Environ. Med. 2004). As a by-product of incomplete combustion there are numerous and diverse sources of formaldehyde. It is produced by automobiles, trucks, and industry, and is released into the environment by natural processes. (EPA air toxics website).

Typical industrial uses for formaldehyde include resins, molding compounds, photographic film, decorative laminates, plywood, and tissue preservative. Not only is HCHO produced in industry but it is also emitted naturally. Natural sources of formaldehyde include forest fires, animal wastes, plant volatiles, microbial processes in biological systems, and it is even produced within our own bodies as a by-product of our metabolism (EPA air toxics website). Although various sources emit HCHO, combustion processes account for most of the formaldehyde that enters into the environment. A primary source of HCHO in ambient air is its direct emission from the incomplete combustion of diesel and gasoline-powered motor vehicles. Formaldehyde is also produced in the atmosphere by the photochemical oxidation of hydrocarbons (VOCs). This secondary source is responsible for the majority of the ambient HCHO. Thus, formaldehyde is an important component of atmospheric pollution (Chen et al. 2004).

Although formaldehyde is ubiquitous in ambient air, the concentrations are relatively low because it is photolyzed and rapidly attacked by the hydroxyl radical. Of special consideration and concern is the HCHO present in home and occupational environments. Indoor air is a significant reservoir of HCHO and in some cases the concentrations can be considerable. The highest concentrations found in indoor air are generally associated with occupational environments and industries that produce HCHO or HCHO-containing products. Also home environments, especially mobile homes, tend to have higher concentrations of HCHO than ambient air (W.H.O. see appendix 5.1, Table 1). Most of this HCHO is from the use of products containing plastics, wood paneling, textiles, carpet, household cleaners, wood stoves, gas burners, and tobacco smoke (TOx probe). Even though different HCHO sources will lead to various exposure concentrations, when evaluating safety and attempting to minimize exposures, perhaps what is most important to consider is the scientific community's current stance that, "based on the review of chamber, community, and workplace studies of human exposure, it is not possible to identify a specific no observed adverse effect level or lowest observed adverse effect level for formaldehyde" (Bender et al).

## **1.2 Significance of Further Formaldehyde Research - Cancer**

For decades researchers have investigated formaldehyde and its potential health effects. It is among the most extensively studied industrial chemicals and there is abundant information from chronic animal inhalation studies and research regarding the carcinogenic potential of formaldehyde. The EPA considers formaldehyde to be a probable human carcinogen and has ranked it in EPA's group B1. Animal studies have reported an increased incidence of nasal squamous cell carcinomas by inhalation exposure (EPA air toxics website). Based on animal inhalation studies, investigators believe that HCHO induced nasal cancer is caused by HCHO's ability to form DNA-protein cross-links in the nasal respiratory mucosa of rats and monkeys. Although genetic effects of HCHO in humans is not well known, *in vitro* human cell studies reveal that HCHO induces DNA damage through DNA-protein cross-links, DNA single-strand breaks, chromosomal aberrations, sister chromatid exchanges, and genetic mutations (TOx Probe).

## **1.3 Significance of Further Formaldehyde Research – Sensory Irritation**

Due to HCHO's various sources and ubiquity, people are likely exposed to low levels of formaldehyde on a daily basis. It has therefore been suggested that formaldehyde exposure may be linked to increased hospital admissions and increased prevalence of inflammatory diseases. Sensory irritation, for example, is a well-known effect of formaldehyde exposure. Some studies have reported that exposure to formaldehyde elicits a variety of allergic signs and symptoms and irritates the upper respiratory tract. These non-cancerous health effects include concentration-dependent eye, nose, and throat irritation; wheezing, coughing, fatigue,

skin rash, nausea, headaches, and difficulty breathing. Although scientific literature is inundated with epidemiologic, community, workplace, and controlled exposure studies, these are mainly observations in human studies concerning eye, nose, and throat irritation (Bender et al.). To date, endpoints other than cancer and sensory irritation are not well characterized; therefore the effects of inhaled formaldehyde on specific inflammatory mediator production are largely unknown. However, one study by Kim et al (2002) reported formaldehyde as a modulating factor of inflammation. This study showed that HCHO has the ability to cause the release of pro-inflammatory cytokines such as IL-1 and IL-8 from dermal keratinocytes, as well as IL-4 and IL-6 from mast cells that lead to clinical deterioration of atopic eczema.

#### **1.4 Cellular Inflammation End-points**

There is limited data to evaluate HCHO's non-cancerous health effects. Cellular inflammation, an important non-cancerous health effect, is a typical response to injury from inhaled pollutants and tends to be characterized by specific mediator production. Many diseases with an inflammatory component are associated with the production of cytokines. In the pathogenesis of respiratory diseases involving inflammation typically irritation and mucus production are preceded by an increase in cytokine release from airway epithelium. Cytokines, such as IL-6 and IL-8, can function to stimulate immune responses to trauma by initiating and amplifying the inflammatory cascade. Cytokine production signals the migration of macrophages and neutrophils to the site of injury and may induce wound repair (Hooper et al.). In addition to being beneficial, cytokines can also have a deleterious effect on the host. Acute and chronic inflammations, as well as thrombosis are among the pathologic conditions that have a significant cytokine component (Hooper et al.). *In vitro* cellular exposure methods have been developed to allow for sensitive assays to evaluate mediators of inflammation. In this paper, cytokines IL-6 and IL-8 were investigated. These cytokines are known to perform an integral function in innate immune responses, inflammation, and perhaps allergic respiratory diseases.

#### **1.5 Study Objective**

The purpose of this study was to develop additional experimental data concerning non-cancerous end-points by measuring markers of inflammation released upon formaldehyde exposure. Cultured human respiratory epithelial cells were used as the target of the formaldehyde gas and formaldehyde was administered in increasing doses to investigate a dose relationship.

## **2 Materials and Methods**

### **2.1 Lung Cell Characteristics**

Type A549 cells were used throughout this study as the biological target of formaldehyde gas exposures. They are alveolar basal epithelial cells that have retained several alveolar type II-cells characteristics, and are derived from an adenocarcinoma. Typically, inhaled pollutants target the epithelial cells lining the alveolar region. Based on study outcomes over the past few years, it has become apparent that alveolar epithelial cells (AECs) are not merely targets for inflammatory mediators, but actively participate in the inflammatory response (Dos Santos et al.). Thus, A549 cells make a good model for studying exposure to inhaled formaldehyde.

In preparation for cell exposures, A549 cells were grown on membranous support (Costar-Clear Transwell inserts; Costar, Cambridge, MA) as described by Jaspers et al. (1997) in complete medium (F12K, 10% fetal bovine serum, antibiotics; all from Invitrogen, Carlsbad, CA) on apical and basolateral sides of the membranous support. Upon confluency, the medium was exchanged for serum-free medium (F12K, 1.5 [micro]g/mL bovine serum albumin (BSA), antibiotics; all from Invitrogen) several hours before exposure (Doyle et al). Immediately preceding exposures to formaldehyde the serum-free media was aspirated and replaced only in the basolateral compartment. Using only basolateral media for the cellular exposures enables direct exposure of cells to formaldehyde gas without media covering the cell significantly interfering with the exposure. Basolateral supernatants are collected once the exposure and 24 hour incubation period has terminated, and stored at -80 °C until they can be analyzed for cytotoxicity and inflammation.

### **2.2 Formaldehyde-Air Mixture Generation and Exposure**

Formaldehyde gas was delivered to A549 cells grown on tissue culture inserts by two methods. We used two delivery systems to expose cells to formaldehyde because we wanted to compare the health effect results from each system. This comparison would not only indicate to us the efficacy of our methods, but if the results were similar for each exposure method, than we could have more confidence in our data and increase the validity and reliability of our results. The schematic in Figure 1 shows the first *in vitro* exposure method that involved using a permeation tube system or primary chamber source in conjunction with humidified dilution air to deliver varying concentrations of formaldehyde to the cells. The

permeation tube generation rate was tracked throughout the study by regular mass-loss determinations (appendix 5.2). Comparisons of calibrations based on the two sources of formaldehyde were made and found to be in agreement. The second method shown by the schematic in Figure 2 involved injecting a specific concentration of formaldehyde (subliming a weighed amount of para-formaldehyde, 95%, using a gently-heated U-tube) into the environmental chamber and then sampling continuously at one liter per minute into the *in vitro* system. These methods are described in more detail below.

### **2.2.1 In vitro Exposure System**

For both exposure methods, cells were maintained in an incubator that houses the *in vitro* cell exposure system. The *in vitro* exposure system maintained the cells at a constant temperature of 37 °C and supplied 5% CO<sub>2</sub> in the air stream throughout the duration of each exposure (Doyle et al).

Inside the incubator, cells grown on tissue culture inserts were placed in an 8-liter, modular, cell-exposure chamber (Billups-Rothenberg, MIC-101™). The 8-liter cell-exposure chamber also has connections for flowing gas through either the permeation tube system or the outdoor chamber. Sample lines through externally circulated sample manifolds were used to provide formaldehyde gas to the cells during exposure (Sexton et al., 2003)-Doyle. Humidified medical-grade air from a cylinder or chamber air was blended with CO<sub>2</sub> at 0.05 liter per minute to achieve about 5% CO<sub>2</sub> in the incubator. This was necessary to maintain the buffering capacity of the tissue culture media under the cells. The addition of CO<sub>2</sub> to chamber air was achieved using small pumps on the exhaust side and mass flow controllers (AALBORG).

### **2.2.2 Permeation Tube System**

For permeation tube exposures a humidification system was developed to humidify the dry formaldehyde gas in air source by humidifying dilution air mixed with source air. This method permits dry air to be used with the permeation tube, and it dilutes the concentration of formaldehyde from the fixed mass emitted by the permeation tubes. Clean air from the ADDCO 737-250 pure air generator was used for both the permeation tube source and dilution air. The dilution air was controlled by a mass flow controller and allowed to bubble through two thermostatically heated midget impingers (ACE Glass, Inc) filled with HPLC grade water (Fisher Scientific). The humidified air was delivered to a mixing tee using thermostatically heated lines to prevent condensation and was blended with the dry formaldehyde-rich air from the permeation tube source. The dew point of the final mixture was measured with a dew point meter (EG&G). Different permeation tubes with different source strengths were used to achieve the desired concentrations in combination with dry

dilution air for the permeation tube source. Figure 1 illustrates how formaldehyde gas leaves the permeation tube system, mixes with both dilution air and humidified air, and is then vented or flushed through the *in vitro* chamber where A549 cells are exposed to the formaldehyde gas. Weight loss histories of the permeation tubes were recorded to determine their source strength (appendix 5.2). In addition, these were compared with standards prepared by injections of weighed amounts of para-formaldehyde into the chamber, as discussed earlier. For concentrations that were within the response range of the method, before most exposures, the Dasgupta method was used to check the formaldehyde concentrations at the outlet and also used to compare the chamber prepared standards measurement responses to the permeation tube measurement responses.

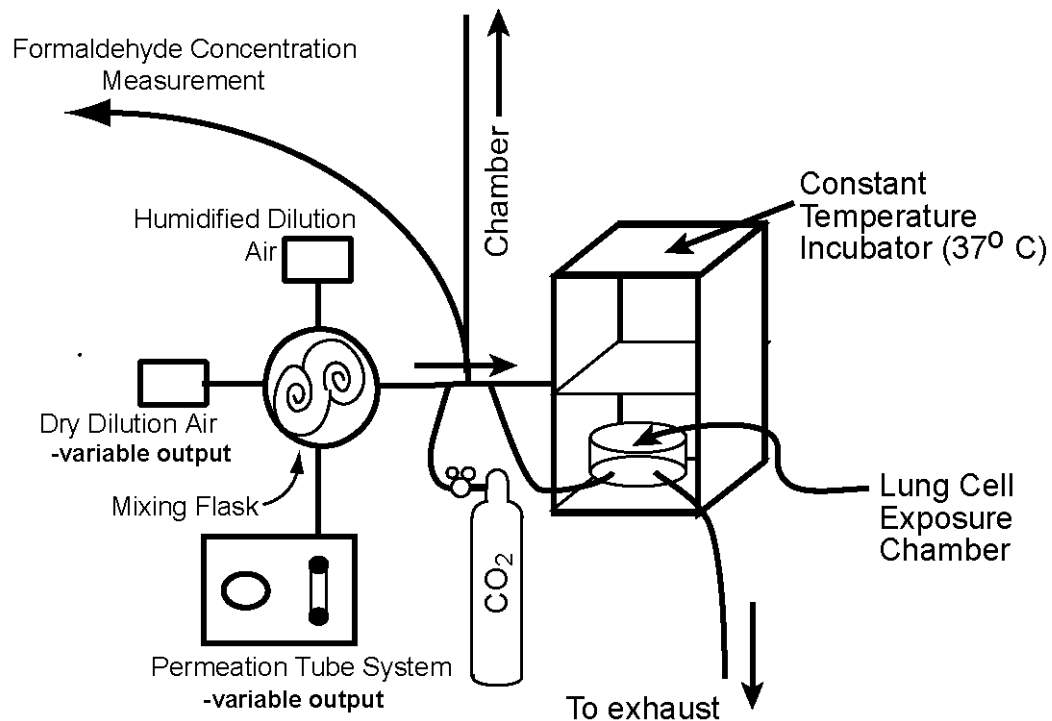


Figure 1: Schematic of Permeation Tube System

Preliminary permeation tube experiments exposed cells to formaldehyde concentrations of 55 ppb, 140 ppb and 220 ppb. We performed these initial experiments to help determine ideal formaldehyde concentrations and exposure durations that might elicit health effects. Except for one IL-8 response performed at 140 ppb, preliminary results showed no significant changes between exposed cells and air-exposed control cells at fairly low concentrations ranging from 55 to 220 ppb (appendix 5.3.1 and 0). Based on these results we started using only higher concentrations of 400 ppb and 1000 ppb and exposure

durations of three hours. The concentrations, at which results are being reported, although high for ambient air levels of formaldehyde, are in the range of possible human exposures. In this case, concentrations reported are among those found in daily exposures living in mobile homes, industrial exposures, and even exposure to tobacco smoke.

On December 23, 2007, January 11, 2008, and February 18, 2008 permeation tube experiments were performed at concentrations of 400 ppb and 1000 ppb. Within same-day experiments, formaldehyde exposures were performed twice with a new set of confluent A549 cells to test the reproducibility of the system. In addition, on each day of an experiment, a clean air control exposure was performed as a point comparison for the cells exposed to formaldehyde gas. All exposures were three hours in duration. For each formaldehyde exposure n=4 sample inserts were used and n=3 sample inserts were used per each clean air control.

### **2.2.3 “One-Atmosphere” Outdoor Environmental Chamber**

Air chemistry research groups at the University of North Carolina at Chapel Hill have been using outdoor irradiation chambers for over 30 years to characterize urban air pollutants and their reactions in ambient air. For a portion of this research, the One-Atmosphere Outdoor Chamber (also called an environmental irradiation chamber or smog chamber) was used as a delivery mechanism for formaldehyde gas. The smog chamber is located on the roof of McGavran-Greenberg Hall on the University of North Carolina at Chapel Hill main campus. It is located directly above the One-Atmosphere Research Lab (on the forth floor of McGavran-Greenberg) where through-the-ceiling/roof sample ports allow gaseous pollutants to be injected into the chamber and samples from the chamber to be delivered to the *in vitro* cell exposure system. The smog chamber is approx 18 ft by 20 ft and has a 120,000-L volume. It has a metal frame and is lined on all sides by a 5-mil thick FEP (fluorinated ethylene propylene) Teflon film that allows for greater than 90% transmission of UV light.



### Outdoor Chamber Schematic

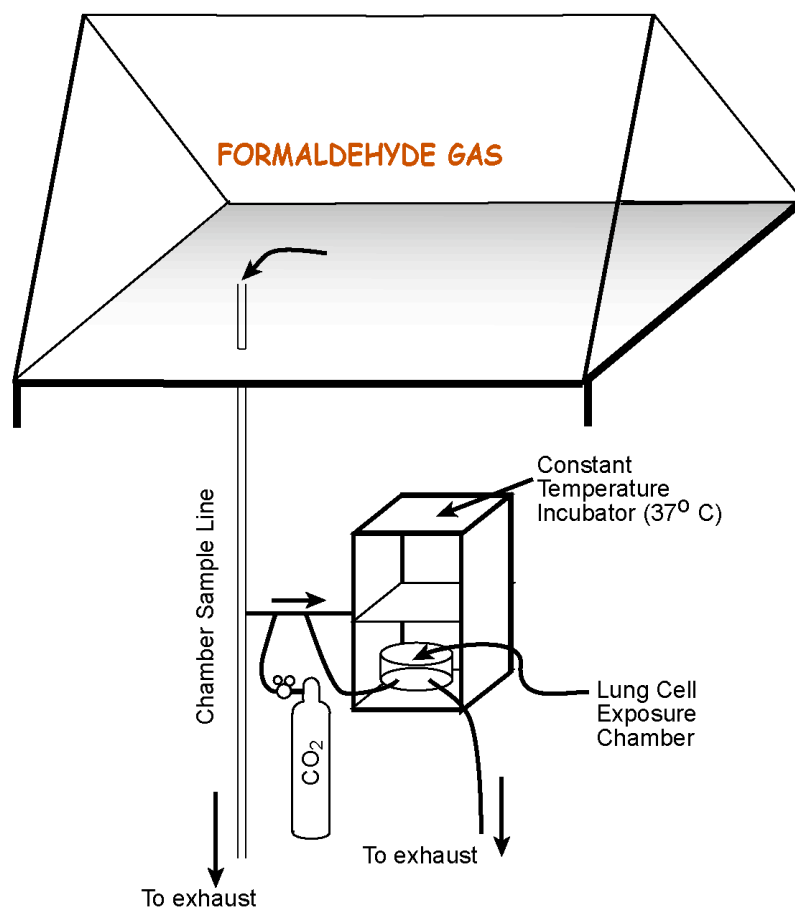


Figure 2: Schematic of Outdoor Smog Chamber

During the chamber September 22, 2007 experiment, formaldehyde gas was delivered to the *in vitro* cell exposure system at two different concentrations. In addition, an incubator control and a clean air control were performed on the same night as the formaldehyde exposure. All exposures were three-hours in duration and performed at night to keep the formaldehyde concentration constant, i.e., no photolysis. For the first exposure, formaldehyde was injected into the chamber at a concentration of approximately 400 ppb. The second exposure used a formaldehyde concentration of approximately 1000 ppb. For each formaldehyde exposure, n=6 sample inserts were used. For each control exposure (clean air and incubator) n=3 sample inserts were used. To prevent condensation inside the chamber after sundown, we used the chamber dehumidification system to lower the dew point below the expected lowest temperature. Condensation would cause HCHO loss due to adsorption into the moisture on the walls. In each experiment, a very small amount (2.5 microliters) of carbon tetrachloride (CCl<sub>4</sub>) used as a tracer, was injected then monitored to calculate the dilution within the chamber (Doyle). The air within the chamber was humidified to 17.3 °C

and the integrity of the cells was maintained by the in vitro cell exposure incubator, keeping the cells at 37 °C and supplied with 5% CO<sub>2</sub>. The dilution rate was measured, and was between 1-2% per hour.

### **2.3 *Quantification of Formaldehyde in Air Mixtures: Primary Sources and Chemical Analysis***

Formaldehyde concentrations were measured continuously using a permeable membrane sampler, and detected with fluorometry using an automated Hantzsch derivative reaction (Dasgupta et al. 1988) for concentrations less than 400 ppb. The purpose of continuously monitoring when possible is to confirm the stability of the formaldehyde sources. Given the history of performance and the primary-standard quality of the permeation tube source and the chamber standards stability in the absence of sunlight, the stability check is for detection of failures or leaks in the sample and delivery lines from the sources. The concentrations in the 3-hour exposures were approximately 55 ppb, 140 ppb, 220 ppb, 390 ppb, and 980 ppb.

### **2.4 *Characterization of Formaldehyde Uptake***

Because formaldehyde is a very water soluble and reactive gas, it is important to measure the concentration losses of formaldehyde to the walls and other surfaces inherent to the exposure system. Formaldehyde loss to tubing, the surface of the exposure system, and media was investigated at a formaldehyde concentration of 220 ppb. This concentration was used because we were able to characterize small losses in concentration with the Dasgupta instrument. Using a weight-loss history of the three permeation tubes, we estimated that without any formaldehyde loss, 600ng of HCHO/min would be delivered through the exposure system.

To evaluate how much formaldehyde was being lost to system walls and tubing only, we used our estimate of how much formaldehyde was going in, and measured what concentration was actually coming from the system outlet. No media or humidifying water dish was used in the exposure system because we did not want to influence surface and tubing loss measurements with any additional formaldehyde sinks. For this exposure the conditions were a dew point of 17.5 °C, an incubator temperature of 37 °C, with an RH of 31%. For the previous conditions and based on our estimates we observed a 6% decrease in formaldehyde, and thus a 6% loss of formaldehyde to the tubing and surface walls of the system.

During actual cell exposures, each Transwell is supplied with 1-mL of media during the exposure to facilitate nutrient supply and prevent the cells from desiccating. Therefore,

we hypothesized that there could also be formaldehyde loss to the sample media and we measured this loss as well. To measure formaldehyde loss to the media, we transferred 1-ml of media into each well of two Transwells and “exposed” this media (in the absence of cells) to formaldehyde for three hours. We then used the Dasgupta instrumentation to directly measure the concentration of formaldehyde in the media. Based on our results we observed approximately 4.5% of HCHO is lost to 1-mL of media. This correlates to 1.62-ug HCHO loss per 1-mL media.

In addition, a common practice with chamber exposures is to include a water dish within the incubator to humidify the air/chemical mix going to the cells. The water dish is a further precaution used to also prevent the cells from desiccating. Although we did not use a water dish in our formaldehyde experiments, we wanted to determine how much formaldehyde could be lost to a dish containing 30-mL of water.

We sampled formaldehyde uptake through the water dish by “exposing” formaldehyde through the system for three hours. We then used the Dasgupta instrumentation to directly measure the concentration of formaldehyde in the water. Based on our results we observed about 13% of HCHO is lost to 30-mL of water (if a water dish is used). This correlates to about 0.43% HCHO loss per mL of water and 0.156-ug HCHO loss per mL of water.

Wall uptake will always occur, however, based on how many Transwells (samples) and thus milliliters of media used formaldehyde loss due to uptake will vary. We measured a 6% uptake of formaldehyde to the walls of the system and a 4.5% uptake of formaldehyde per mL of media used. Exposures typically use three to four mL of media. Based on our measurements, this leaves between 76% and 81% of the original concentration of formaldehyde available for exposure (appendix 5.4).

## **2.5 Health Effects Analysis**

When cells become stressed or damaged, multiple genes and signaling pathways are stimulated. This causes various proteins to be produced in the cell and then subsequently released into the cells’ environment. Proteins, like mediators of inflammation, have a diverse ensemble of function and can both assist the respiratory system or eventually cause respiratory disease. Mediators of inflammation can minimize damage by recruiting cells to provide a physical barrier to further damage. This cell recruitment may also constitute the structural basis for mucociliary clearance (Hiemstra et al.). Although these mechanisms are important to maintain the structure and function of the respiratory tract, they may also contribute to the development of respiratory disease. Mediators of inflammation can cause acute and chronic inflammation and activation of airway epithelial cells is associated with

asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and respiratory infection (Hiemstra et al.). An additional health effect of respiratory trauma is cellular death, which can be measured by the release of the enzyme lactate dehydrogenase.

After clean air and formaldehyde exposures, cells were incubated at 37 °C for 24 hours. Basolateral supernatants were collected and stored at -80 degrees C until analysis of cytokines and markers of cytotoxicity could be performed.

## **2.6 Cell Cytotoxicity**

Basolateral supernatants previously stored at -80 °C were used in the analysis of cytotoxicity. Cytotoxicity was examined by measuring the release of lactate dehydrogenase (LDH) using a coupled enzymatic assay followed by manufacturer's instruction (Takara, Japan). LDH is an enzyme that is strictly produced and maintained within a cell, thus release of LDH into the supernatant is considered to be a marker of cell membrane disruption and consequently cell death.

### **2.6.1 ELISA - IL-8 and IL-6 Expression**

Cellular trauma stimulates intracellular signaling cascades that cause pro-inflammatory cytokines to be produced. Pro-inflammatory cytokines/chemokines are chemical signals that attract neutrophils and other inflammatory cells to the site of trauma, leading to inflammation. The inflammatory responses of A549 cells upon formaldehyde exposure were quantified using an Enzyme-Linked ImmunoSorbent Assay (ELISA) kit. Basolateral supernatants previously stored at -80 °C were used in the analyses of markers of inflammation. Protein levels of pro-inflammatory cytokines interleukin-8 (IL-8), and Interleukin-6 (IL-6) were measured by ELISA (R&D Systems, Minneapolis, MN), as per the supplier's instructions.

Interleukin-6 (IL-6) acts as both a pro-inflammatory and anti-inflammatory cytokine. It functions to stimulate immune responses to trauma. In terms of host response to a foreign pathogen, IL-6 has been shown, in mice, to be required for resistance against bacterium. IL-8 is a chemokine and it belongs to a group of CXC chemokines characterized by two N-terminal cysteine residues separated by one non-conserved amino acid residue at the N-terminal end. (Rudack et al.) Besides initiating the adhesion cascade, IL-8 is known to be a potent neutrophil chemotactic protein. IL-8 recruits migrating neutrophils and leukocytes to areas of inflammation. Neutrophils infiltrate the site of injury and function to phagocytose the antigen that triggered the initial inflammatory response. IL-8 is believed to play a role in the pathogenesis of bronchiolitis, a common respiratory tract disease caused by viral infection. It has been implicated as an important mediator of neutrophil infiltration in lung

tissues and thus serves to amplify the inflammatory cascade. IL-8 is produced in response to TNF- $\alpha$  in primary cultured human airway epithelial cells and in a human lung epithelial cell line (Li et al.)

### **2.6.2 Multi-cytokine Antibody Array**

A preliminary effort in understanding potential mechanistic effects of HCHO exposure was to perform a multi-cytokine antibody array. The multi-cytokine detection assay used basolateral supernatants previously stored at -80 ° C from the HCHO chamber exposure performed on September 22, 2007. During this experiment three exposure conditions were performed: a three hour incubator/clean air control (n=6), a three hour 400 ppb HCHO exposure (n=6), and a three hour 1000 ppb HCHO exposure (n=6). The array examined the basolateral supernatants for 13 cytokines/chemokines. Only RANTES and MCP-1 were detectable. All other cytokines were either below the limit of detection or below background levels.

When comparing the HCHO exposures to the controls for both RANTES and MCP-1 there were no statistical differences between controls or exposures, however trends were apparent. See appendix 5.5.

### **2.7 Statistical Analysis**

To analyze our data, one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test was used as a statistical test to determine the significance. ANOVA was the most appropriate statistical test to use due to the need for multiple comparisons. Generally, during each experiment, three or more data sets were produced and it was necessary to compare these data sets to one another. A value of  $p \leq 0.05$  was considered to be significant.

### **3 Results**

In this study, as indicators of deleterious cellular effects, IL-8 and IL-6 gene expression along with cytotoxicity were used to estimate the health effects elicited from gaseous formaldehyde exposure. A549 cells grown to confluency on membranous supports were exposed for 3 hours to formaldehyde gas at concentrations of 400 ppb and 1000 ppb. The results from the experiments performed were not only reproducible within the same formaldehyde delivery system, but also when comparing permeation tube exposures and chamber exposures.

#### **3.1 *Permeation Tube Exposure Results***

The permeation tube exposures of 400 ppb lasting three hours (combined responses from both exposures) were evaluated for their potential to cause adverse cellular damage to the A549 cells. Figure 3, Figure 4, and Figure 5 show that by our end-points, there were no significant differences between air-and formaldehyde-induced IL-8, IL-6 expression or cytotoxicity. The permeation tube exposures of 1000 ppb lasting three hours (combined responses from both exposures) were also analyzed for cell death and markers of inflammation. We observed similar results to the 400 ppb exposures where our analysis showed no difference in IL-6 expression or in cell death (LDH). See Figure 4 and Figure 5. However, unlike the 400 ppb exposure, a significant decrease ( $p\text{-value} \leq 0.05$ ) in IL-8 release was observed with the 1000 ppb exposure. Figure 3 shows that this suppression was not only significant to its experimental control, but also significantly less than the 400 ppb exposure when comparing the fold change of each experiment over their controls ( $p\text{-value} \leq 0.002$ ).

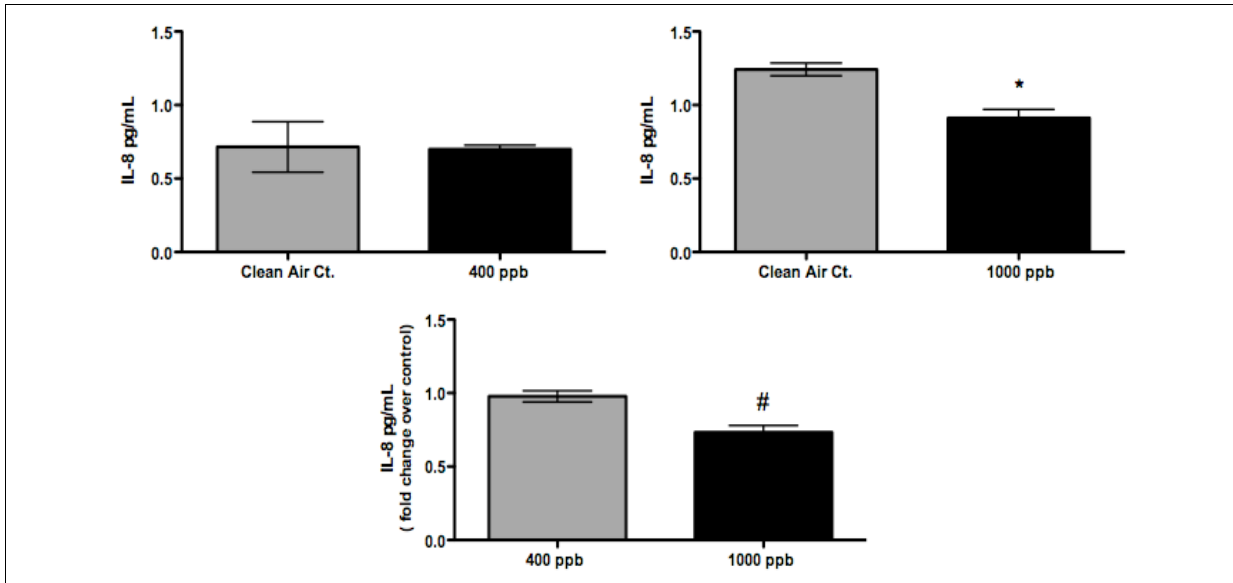


Figure 3: IL-8 responses from cell exposures using the Permeation Tubes. No difference in IL-8 response for formaldehyde concentrations up to 400 ppb. At the 1000 ppb level we observed a significant decrease in IL-8. [Symbols: \*significantly different from control ( $p \leq 0.05$ ); # significantly different from samples exposed to 400 ppb ( $p \leq 0.05$ )]

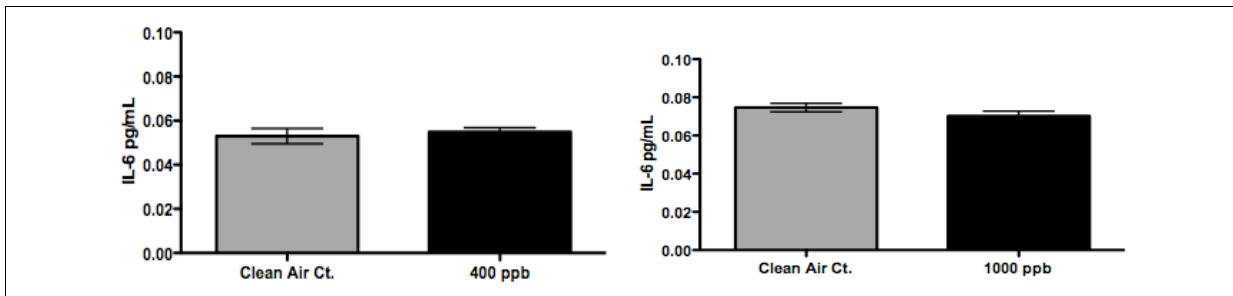


Figure 4: IL-6 responses from cell exposures using the Permeation Tubes. No differences in IL-6 response for formaldehyde concentrations up to 1000 ppb were observed. [Symbols: \*significantly different from control ( $p \leq 0.05$ )]

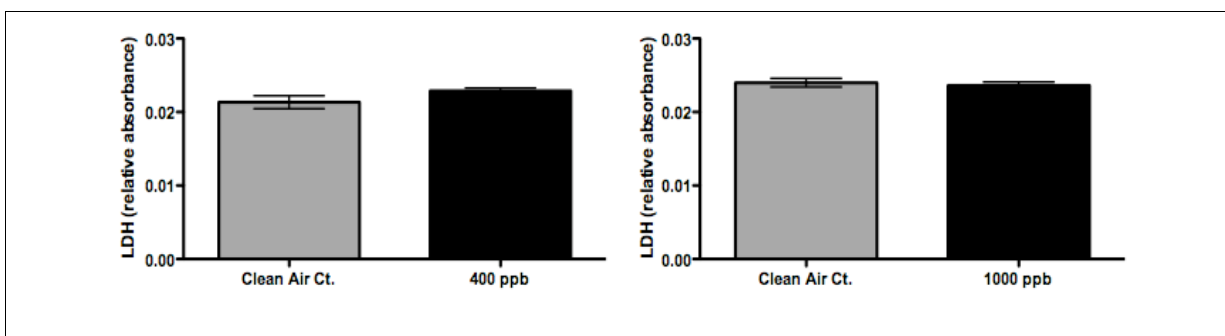


Figure 5: Figure 3: LDH release (cell death) from cell exposures using the Permeation Tube. Neither the 400 ppb nor 1000 ppb concentration showed a significant change from the clean air control.

### 3.2 Chamber Exposure Results

A549 cells exposed to chamber clean air and to formaldehyde injected into the chamber at concentrations of 400 ppb and 1000 ppb were also analyzed for cell death and markers of inflammation. In the case of the chamber experiment, the three hour - 400 ppb exposure was performed first and then the chamber concentration of formaldehyde was brought up to 1000 ppb for another three hour exposure. For IL-6 and LDH no significant difference between formaldehyde exposed cells and clean air exposed cells was observed for either the 400 ppb or 1000 ppb exposure. However, analysis of IL-8 revealed a step-wise concentration dependent suppression of IL-8 release (see Figure 6).

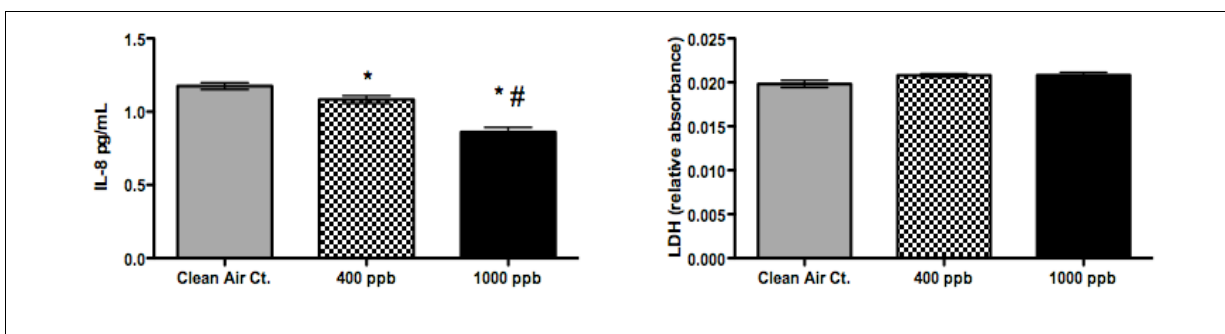


Figure 6: IL-8 responses and LDH release (cell death) from cell exposures using the outdoor chamber. A concentration dependent step-wise decrease in IL-8 is observed. Although there was a significant decrease in IL-8 response from controls and between samples, no increase in cell death was observed with increasing concentrations of formaldehyde. [Symbols: \*significantly different from control; # significantly different from samples exposed to 400 ppb ( $p \leq 0.05$ )



## 4 Discussion and Conclusions

A literature review suggests that no previous studies have used a coupled chamber-*in vitro* cell exposure system to deliver formaldehyde gas, the inflammatory agent, by continuously flowing it over lung cells for predetermined exposure duration. Studies typically inject or bubble HCHO directly into media that contains lung epithelial cells. Our exposure method effectively mimics the actual exposure of gases flowing through and over an epithelial organ system. Based on our research an initial observation is that formaldehyde exposure to A549 cells caused no increases in LDH or IL-6. Alone, this could indicate that either formaldehyde has no affect on LDH and IL-6 from alveolar epithelial lung cells, or our *in vitro* delivery system was not an efficient exposure method for formaldehyde, however, additional evidence from this study leads to the contrary. Throughout chamber and permeation tube exposures, a recurring event of a decrease in IL-8 expression suggests that our system is in fact effectively delivering formaldehyde gas to the *in vitro* cell exposure system. Based on the decrease in IL-8 we believe that formaldehyde is having some mechanistic effect on specific inflammatory mediator production. Therefore, the overall finding of this study is that while LDH and IL-6 levels remain unchanged with regard to exposed samples and control samples, IL-8 expression decreases. The main focus of this discussion will therefore be on IL-8 expression and its function not only in epithelial cells, but in other cells types as well.

Interleukin-8 (IL-8) is a pro-inflammatory cytokine and a member of the CXC chemokine family. It is the most-well-known and characterized neutrophil-attracting chemokine (Rudack et al.). It is produced by a wide variety of cell types including mononuclear cells, endothelial cells, and epithelial cells. Through a chain of biochemical reactions IL-8 is secreted and is an important protein involved in innate immune system responses. It functions as a chemoattractant, serving as a chemical signal to recruit neutrophils and leukocytes to the site of injury. In non-inflamed tissue, IL-8 expression is low but can be rapidly induced by pro-inflammatory cytokines. Typically, inflammation and transient IL-8 expression are beneficial (Nozell et al.). Although IL-8 was originally shown to attract and activate neutrophils, and initiate the inflammatory cascade, this chemokine has multiple biological functions. IL-8, as well as other cytokines, can function to induce the acute-phase response, initiate mononuclear cell migration to the site of injury, induce wound repair, and both augment and amplify the immune response (Hooper et al.). In addition to being beneficial, cytokines can also have a deleterious effect on the host. Based on findings from multiple studies, IL-8 has also been implicated as having some involvement with

processes that lead to cancerous endpoints. IL-8 has been shown to induce the migration of some tumor cells, regulate pathological angiogenesis, tumor growth, and metastasis; it is therefore a potent angiogenic factor. Although angiogenesis is a normal process in growth and development, as well as in wound healing, it is also a fundamental step in the transition of tumors from a dormant state to a malignant state.

Not only does IL-8 have multiple functions and is expressed differently depending on stimulant and cell type, but there are multiple signaling cascades that can lead to IL-8 production (Jaspers, 1998). Further evidence is found in a study by Hooper et al. that indicates endothelial cells produce IL-8 as an important physiologic participant in cross talk between coagulation and inflammation. In this study, IL-8 is produced in periodontal tissues where healthy cells always maintain a low level of cellular inflammatory infiltrate. A study by Darveau et al. demonstrated that the expression of several molecular mediators of inflammation is normal and important in healthy tissues. With regard to healthy periodontal tissue, low-level expression of mediators such as IL-8 most likely contribute to the ability of the host to limit periodontal bacterial growth and injury to the tooth and epithelial cell surface (Darveau et al.). IL-8 is also synthesized in ovary cells and a study by Lee et al. provides insight into the possible anti-tumorigenic potential of IL-8. In contrast to other studies, their findings show that tumor growth in vivo was significantly attenuated in animals receiving IL-8-expressing cells when compared with mice injected with control cells. In their model the local production from ovary cells of IL-8, and the subsequent neutrophilic infiltration, result in markedly decreased tumor growth (Lee et al.). It is obvious from these various studies in multiple organ systems that IL-8 performs vital functions for the well being of healthy cell systems. Although IL-8 has many known functions, for the most part, the mechanisms that facilitate IL-8 production are poorly understood. It is also not well known how IL-8 production impacts all the cellular systems that release it.

This overview of the current scientific knowledge surrounding IL-8 reveals that IL-8 has various functions and its production is initiated by various cellular changes. The overall effect of IL-8 appears to require additional factors necessary for metastasis based on the cells in question. In addition, IL-8's cancerous involvement in various tumor cell types may be dependent on the metastatic potentials of the specific cancer cell lines. In terms of cancerous or non-cancer end-points, there appears to be a very fine balance between neutrophils/macrophages, chemotaxis, and angiogenesis/metastasis (Lee et al.).

Most significant to the focus of this paper is IL-8's role and production in the respiratory system. Multiple investigations into respiratory production of IL-8 indicate that it is constitutively expressed and released by nasal, bronchial, and alveolar epithelial cells. It is known to be augmented not only by infectious stress but also by mechanical and chemical stress (Hagimoto et al.). Rudack et al. states that overall IL-8 has an important part in

respiratory defense mechanisms. IL-8 not only functions as a protective response to respiratory insults, but provides maintenance services as well. Within the respiratory track, IL-8 is being constantly synthesized; this results in maintaining neutrophils on the mucosal surface and in restoring the homeostatic balance. Our results indicate a suppression in IL-8 expression. Impairing IL-8 synthesis could have serious repercussions for the overall health of the respiratory system. If in fact IL-8 is integral not only as a protective mechanism, but as a maintenance one as well, exposure to formaldehyde could severely hinder proper functioning of the respiratory system. Although IL-8 expression is associated with deleterious effects such as chronic inflammation and cancer, low level IL-8 expression is vital to repairing and protecting damaged respiratory epithelial cells. Formaldehyde, acting as an IL-8 suppressor, could effectively and indirectly be causing cells to be more vulnerable to inhaled insults. Taken together, with increased vulnerability and a handicapped recovery system, formaldehyde could be causing much more damage than can be assessed by the overall results presented in this study.

#### **4.1 Limitations**

When using an *in vitro* exposure system there are limitations inherent to the method. Generally the system is very isolated because investigators typically want to focus on one cell type's responses to a particular insult. While being able to narrow the scope of an investigation may be an advantage, perhaps in this case, our window is too defined for HCHO exposures using only A549 cells taken outside of their natural environment. It is possible that the A549 cells could respond differently in a comprehensive system using more than one cell type and including other cell-to-cell interactions. Unfortunately with the *in vitro* system we used, we are unable to evaluate cell-to-cell interactions and other potential cell system impacts of HCHO exposure. Ideally one would like to use a larger system because it is more holistic and more realistic, but it is also more technically challenging to probe such a system.

Another limitation of the study is the dose of formaldehyde delivered to the A549 cells. In this study, we exposed A549 cells to specific concentrations of HCHO for three hours. Typically HCHO exposures to an individual would occur over a much more extended and various period. Depending on the situation, an individual could be exposed through the environment to small concentrations over their lifetime, massive acute concentrations if they are a smoker, or if an individual worked in a HCHO producing industry they could potentially be exposed to very high concentrations each workday for up to eight hours.

#### **4.2 Future Research**

Considering the results of this study future research in other mechanistic effects of formaldehyde seems most logical. Using current samples analyzed from this study, it would

be very interesting and beneficial to analyze other genetic markers and cellular responses upon HCHO exposure.

In addition a technically challenging and fairly novel approach to *in vitro* testing would be to use HCHO “conditioned” cell supernatants from A549 cells and incubate that media with another cell type found in the same respiratory environment. Perhaps to see the bigger picture of HCHO induced injury, the A549 cells need to interact with other cells in their actual alveolar environment.

### **4.3 Final Conclusions**

Throughout this study we have emphasized that formaldehyde is a known respiratory irritant and probable human carcinogen. Although formaldehyde exposure has been studied extensively for over thirty years, most of the research has focused on *in vivo* murine models with cancer endpoints. Many studies have investigated formaldehyde’s role in nasal cancer. These studies have made great contributions to understanding the biologic mechanisms of formaldehyde damage at high concentrations. They have concluded that inhaled formaldehyde increases incidence of nasal squamous cell carcinomas through DNA-protein cross-links in murine models (EPA air toxics website). However, due to the increasing trend in allergic respiratory disease and epidemiologic data suggesting a potential link to formaldehyde exposure, there has been an overwhelming need to investigate other endpoints such as inflammation. Formaldehyde has been implicated as a cause of non-cancerous respiratory disease because of its chemistry, ubiquity, prevalence in home and work environments, and the fact that its various sources permeate throughout every socioeconomic group. Using conventional, well-characterized markers of inflammation, a main goal of this study was to generate data on lung epithelial inflammatory responses upon exposure to formaldehyde.

Although mechanistic pathways of HCHO damage to the respiratory system are outside of the scope of this study, our results indicate that HCHO is affecting markers of inflammation in a very atypical fashion. Generally, inflammation is associated with increased cytokine production. However, in this study we see a concentration-dependent decrease in IL-8 release and no effect on IL-6. Although it is possible that our observed effects of HCHO on pro-inflammatory cytokine production could be the result of enhanced production of anti-inflammatory cytokines or a mechanistic inhibition of the TNF- $\alpha$  or NF- $\kappa$ B pathway, further research is necessary. Unfortunately, at this point, a mechanistic cause of HCHO induced IL-8 suppression is unknown; however these results provides further impetus for additional and more focused HCHO research on mechanistic effects.

## 5 Appendices

### 5.1 W.H.O. Formaldehyde Concentrations

Table 1: Sources and average concentrations of formaldehyde in the environment. Source: WHO Regional Office for Europe - Air Quality Guidelines Chapter 5.8 Formaldehyde

Source	Concentration (µg/m <sup>3</sup> )	Concentration (ppb)	Exposure (µg/day)
<b>Ambient air</b> (10% of time; 2m <sup>3</sup> /day)	1.0 - 20	0.814 - 16.3	2.0 - 4.0
<b>Home</b> 65% of time; 10m <sup>3</sup> /day			
<b>Conventional home</b>	30 - 60	24.4 - 48.9	300 - 600
<b>Mobile home</b>	100	81.4	1000
<b>Workplace</b> 25% of time; 8m <sup>3</sup> /day			
<b>Without occupational Exposure</b>	30 - 60	24.4 - 48.9	200 - 500
<b>With occupational Exposure</b>	1000	800	8000
<b>Environmental tobacco smoke</b>	50 - 350	41.0 - 285	400 - 2800
<b>Smoking (20 cigs/day)</b>	60000-130000	48000 - 104000	900 - 2000

### 5.2 Permeation Tube Generation Rate – Weight-loss Histories

The permeation tube generation rate was tracked throughout the study by regular mass-loss determinations. Weight-loss histories of the permeation tubes were recorded to determine each permeation tube's individual source strength.

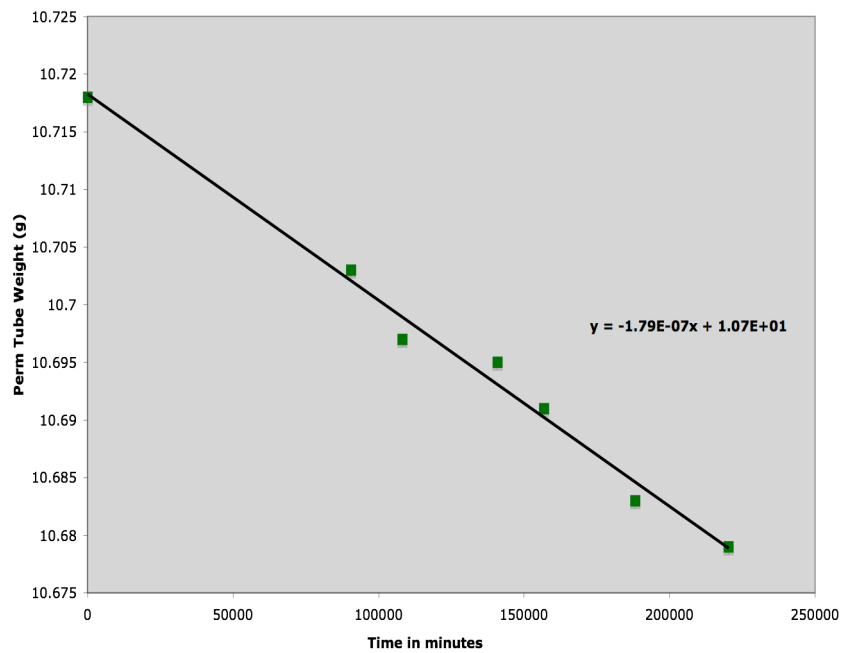


Figure 7: Weight-loss history for permeation tube "89". Concentration output is approximately 55 ppb.

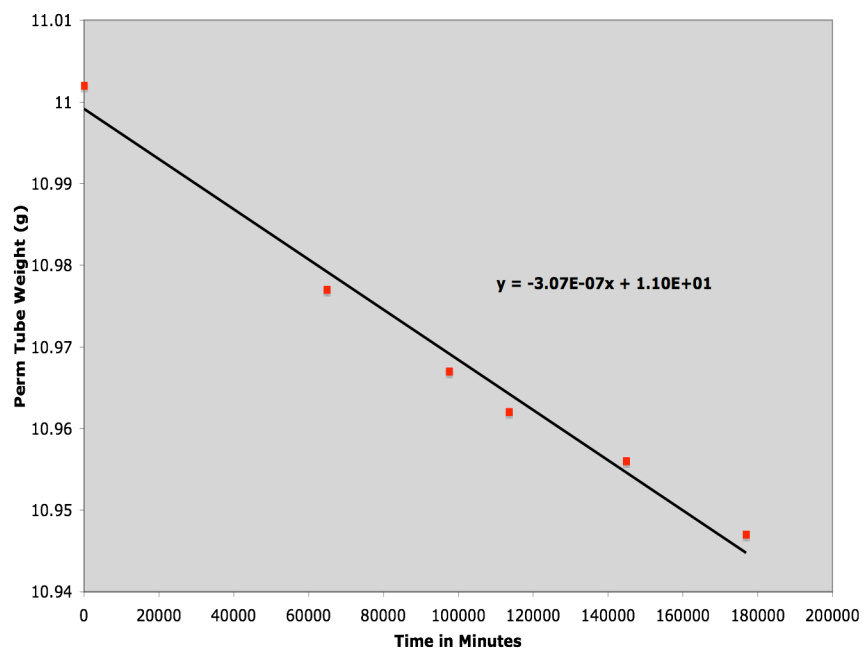


Figure 8: Weight-loss history for permeation tube "88". Concentration output is approximately 85 ppb

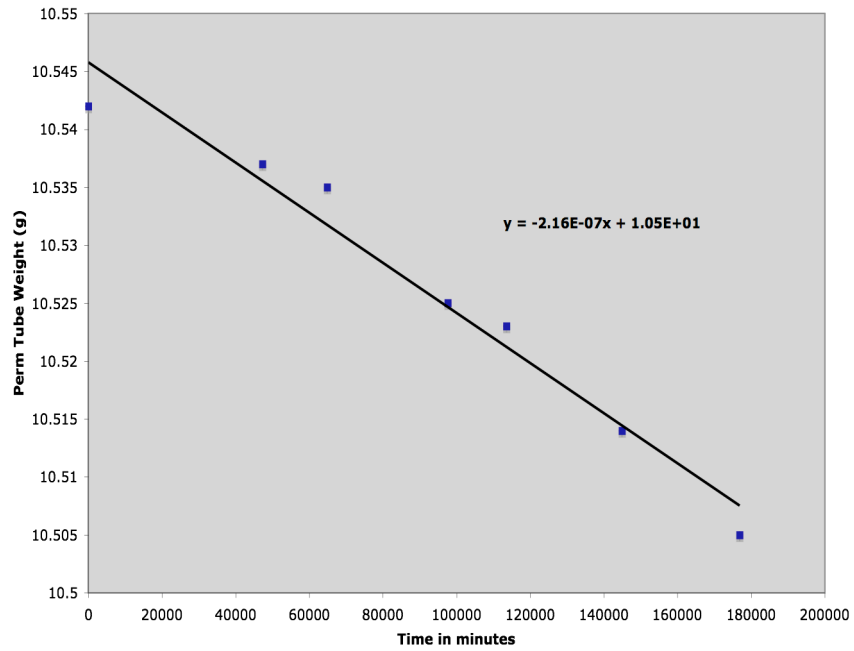


Figure 9: Weight-loss history for permeation tube "85". Concentration output is approximately 80 ppb.

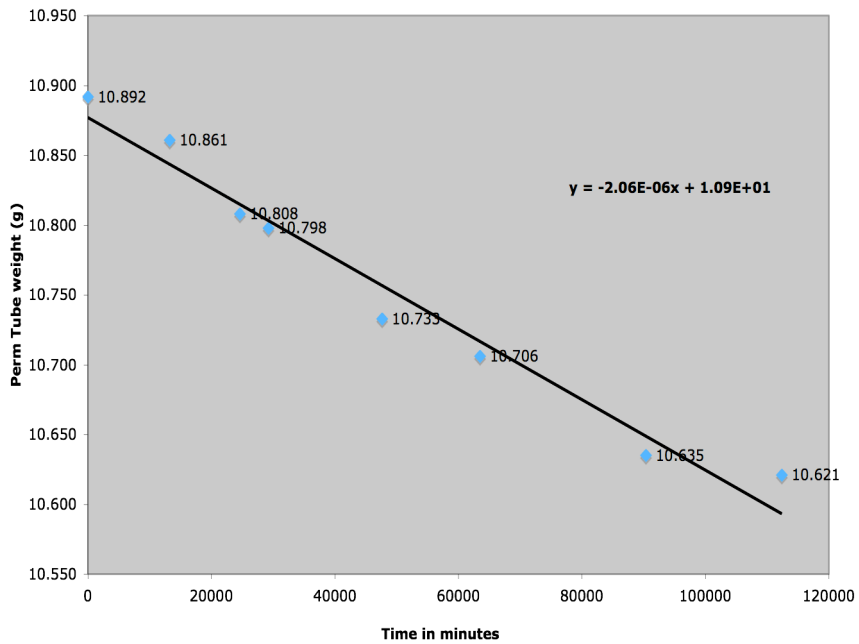


Figure 10: Weight-loss history for "two ppm" permeation tube

### 5.3 Results for Permeation Tube Exposures of 55 ppb to 220 ppb

#### 5.3.1 IL-8 Results for Concentrations 55 ppb, 140 ppb, and 220 ppb

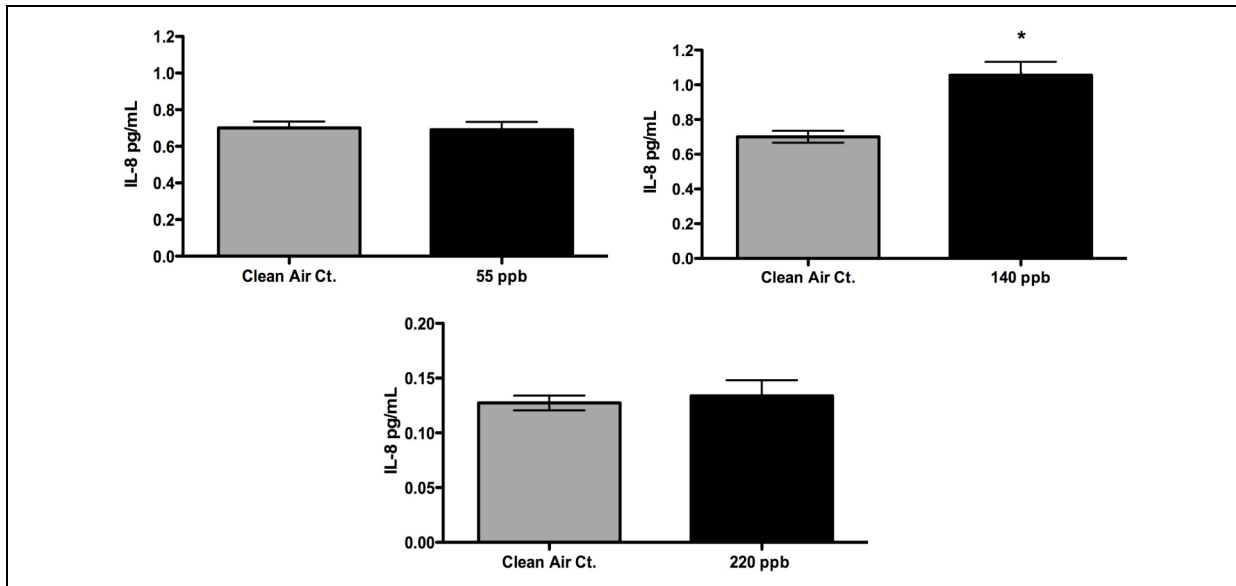


Figure 11: IL-8 results for HCHO exposures of 55 ppb, 140 ppb, and 220 ppb. Only the 140 ppb exposure showed a significant increase in IL-8 expression over its clean air control. [Symbols: \*significantly different from control ( $p \leq 0.05$ )]



### 5.3.2 LDH Results for Concentrations 55 ppb, 140 ppb, and 220 ppb

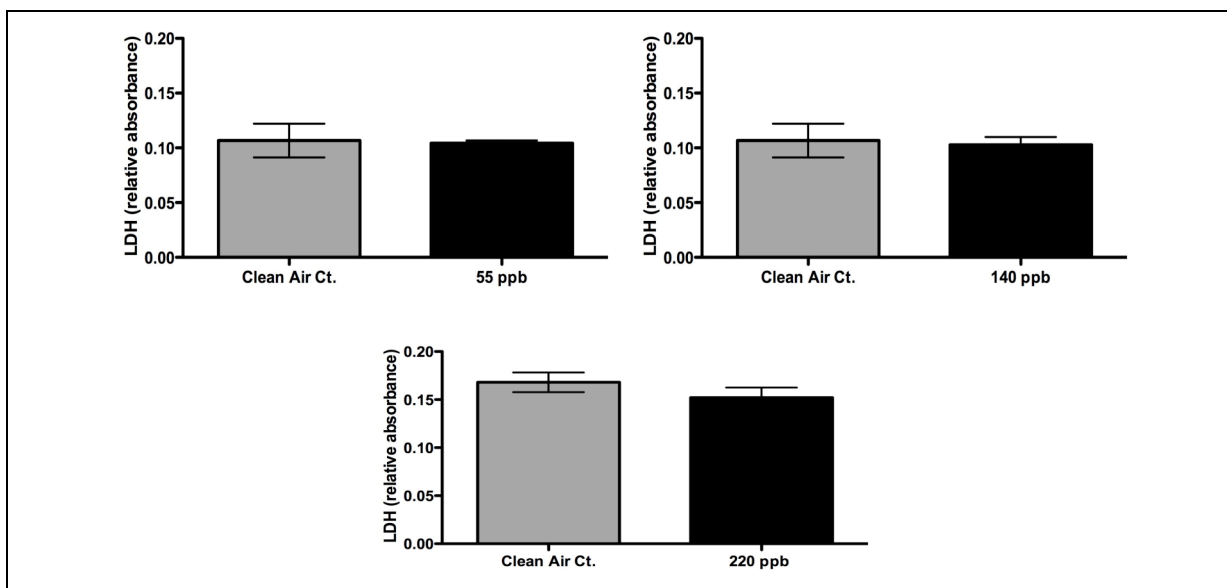


Figure 12: LDH results for HCHO exposures of 55 ppb, 140 ppb, and 220 ppb. No significant differences were found between HCHO exposures and their clean air controls.

### 5.4 Characterization of Formaldehyde Loss

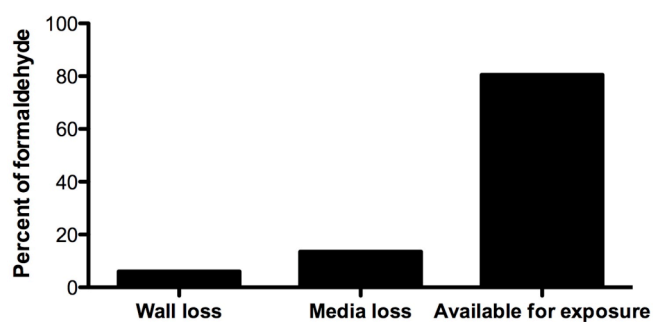


Figure 13: Characterization of Formaldehyde loss. Based on an n=3 sample set, around 80% of the HCHO is available to the cells for exposure.

## 5.5 Multi-cytokine Antibody Array Results

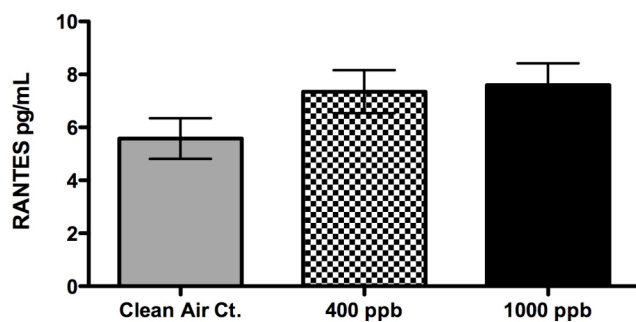


Figure 14: Results from RANTES detection assay. No statistical differences between clean air controls and HCHO exposures, however there is a trend of increased RANTES expression from HCHO exposures.

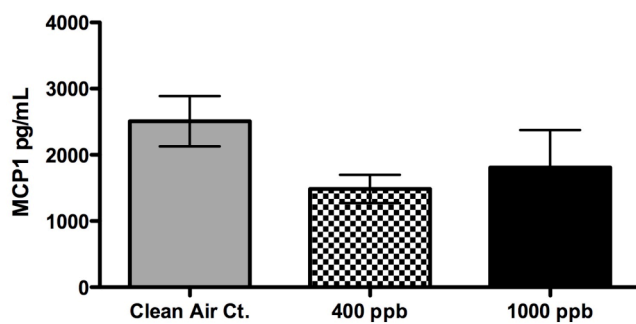


Figure 15: Results from MCP-1 detection assay. No statistical differences between clean air controls and HCHO exposures, however there is a trend of decreased MCP-1 expression from HCHO exposures.

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